From immune phenotype to clinical manifestations in CVID

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Serviço de Imunoalergologia, HSM – CHLN

2015/2016

Faculdade de Medicina da Universidade de Lisboa

Contents

Resumo

Introdução: A Imunodeficiência Comum Variável (IDCV) constitui a causa mais frequente de imunodeficiência primária sintomática, caracterizada por hipogamaglobulinemia resultante de defeitos na diferenciação das células B periféricas e alterações das subpopulações de células T. A sua diversidade imunológica e clínica dificultam a investigação da fisiopatologia subjacente e a identificação de factores de prognóstico.

Material e métodos: Foram consultados os processos clínicos e os dados imunológicos de 60 doentes adultos (idade média 45 ± 13anos; duração média de follow-up de 8.5 anos, máximo 24 anos) em 2015. Focou-se posteriormente a análise num subgrupo de 29 doentes com avaliação clínica e imunológica detalhada em 2008. Além das subpopulações de células B, quantificou-se por citometria de fluxo o grau de activação das células T e a diminuição das células CD4 naïve e comparou-se com controlos saudáveis com idades semelhantes. Compararam-se ainda estes parâmetros em grupos IDCV divididos consoante as suas manifestações clínicas. Correlacionaram-se as manifestações clínicas e as alterações das subpopulações linfocitárias B e T e avaliou-se a estabilidade do fenótipo clínico durante o follow-up.

Resultados: A prevalência actual de manifestações clínicas não-infecciosas é muito elevada, sendo que unicamente 3 doentes apresentam apenas infecções. Não obstante, em 63% dos casos, as manifestações iniciais da IDCV foram infecções respiratórias recorrentes. Citopénias autoimunes, esplenomegália, adenopatias e proliferação linfóide estão associadas a níveis mais elevados de marcadores de activação de células T, perda de células T CD4 naïve e expansão de células B $CD21^{low}CD38^{low}$. Demonstrou-se que o espectro das manifestações clínicas evolui apesar do tratamento de substituição com IgG.

Conclusões: São necessárias terapêuticas adicionais para limitar a progressão de complicações não-infecciosas. Actuar sobre as alterações das células T poderá ser uma estratégia a ser explorada na terapêutica destes doentes.

Abstract

Introduction: Common Variable Immunodeficiency (CVID) represents the most frequent cause of symptomatic primary immunodeficiency, defined by hypogammaglobulinemia due to defects in peripheral B-cell differentiation and disturbances in T-cell subsets. The immunologic and clinical diversity of CVID hampers the discovery of underlying disease-causing mechanisms and clinical or laboratorial relevant prognostic factors.

Material and methods: We reviewed medical records and immunological data of 60 adult patients, (mean age 45 ± 13years; mean length of follow-up 8.5 years, up to 24). We further focused our analysis in a subgroup of 29 patients from whom we have detailed clinical and immunological evaluations performed 7 years before. In addition to the standard B-cell populations, we extended flow-cytometric analysis to quantify the loss of naïve CD4 T-cells and degree of T-cell activation. We compared these parameters in CVID groups split according to the presence of a given clinical manifestation and evaluated the stability of the clinical phenotype, through the analysis of the largest adult cohort under follow-up in a portuguese Centre.

Results: The current prevalence of non-infectious manifestations was remarkably high, and an infection-only profile was confined to 3 patients. Nevertheless, in 63% of the cases, the initial manifestations were recurrent respiratory infections. Autoimmune cytopenias, splenomegaly, adenopathies and lymphoid proliferation were associated with significantly higher levels of T-cell activation markers, naïve CD4 T-cell loss and expansion of $CD21^{low}CD38^{low}$ B-cells. We were able to show that throughout follow-up the spectrum of clinical manifestations expands despite IgG replacement treatment.

Conclusions: Additional therapies are required to contain the emergence of non-infectious complications that are main determinants of morbidity in CVID patients. Our data support that the targeting of T-cell imbalances may be a therapeutic strategy to be explored in the management of these patients.

Introduction

Common variable immunodeficiency (CVID) represents a heterogeneous subset of hypogammaglobulinemias of unknown etiology[1,2] and the most frequent cause of symptomatic primary immunodeficiency[3]. Its frequency is estimated to be 1/25.000-50.000 in the Western European countries[4,5]. There are regional differences in incidence, being a rare diagnosis among Asians and Afro-Americans. There is no gender predisposition and the age of onset is usually around the second to third decade, although first manifestations of CVID may occur at any age[1,2,6,7].

CVID is a primary humoral immunodeficiency disease characterized by quantitative and qualitative reduction in antibody production (hypogammaglobulinemia) due to heterogeneous defects in mature B-cells and T-cells[4,8], that should be considered in any patient older than 4 years[9]. According to the current diagnostic criteria of the European Society for Immunodeficiencies (ESID)[10], CVID is considered probable in a patient who fulfills the following requirements:

1. At least one of the following:
    a. Increased susceptibility to infection
    b. Autoimmune manifestations
    c. Granulomatous disease
    d. Unexplained polyclonal lymphoproliferation
    e. Affected family member with antibody deficiency
2. Marked decrease of immunoglobulin (Ig) G and marked decreased of IgA with or without low IgM levels (measure at least twice; at least 2 standard deviation below the normal levels for the age)
3. At least one of the following:
    a. poor antibody responses to vaccines (and/or absent isohemagglutinins);
    b. low switched memory B-cells (<70% of age-related normal value)
4. Secondary causes of hypogammaglobulinemia have been excluded
5. Diagnosis is established after fourth year of life (but symptoms may be present before)
6. No evidence of profound T-cell deficiency, defined as 2 out of the following:
    a. CD4 counts/μl: 2-6years of life (y) <300, 6-12y <250, >12y <200

b. Frequency of naïve CD4: 2-6y <25%, 6-16y <20%, >16y <10%
c. T-cell proliferation absent

The range of CVID clinical manifestations is broad, including acute and chronic infections, autoimmune and inflammatory diseases and an increased incidence of cancer and lymphoma[6]. Therefore, the disease phenotype is both heterogeneous and complex, which contributes to an average delay of 6-7 years in the diagnosis of this syndrome[6].

As expected, the lack of antibodies which is the main characteristic of CVID, leads to recurrent sinopulmonary tract and gastrointestinal infections[1,6]. These infections are mainly caused by encapsulated bacteria like *Haemophilus influenza*, *Streptococcus pneumonia*, as well as *Moraxella catarrhalis*, different Staphylococci and *Giardia lamblia*[2].

Autoimmunity occurs in approximately 10 to 20% of CVID patients and the most common autoimmune disease related to CVID is primary immune thrombocytopenia (ITP), followed by autoimmune hemolytic anemia (AIHA)[1,3,7]. Other autoimmune conditions as antiphospholipid syndrome, diabetes *mellitus*, inflammatory bowel disease, pernicious anemia, rheumatoid arthritis, uveitis, multiple sclerosis, neutropenia, primary biliary cirrhosis, systemic lupus erythematosus (SLE), autoimmune thyroid disease, vasculitis, psoriasis and vitiligo are also frequently associated with CVID[3,7,11].

The inflammatory phenotype in CVID is characterized by polyclonal lymphocyte infiltration followed by fibrosis and eventually granuloma formation[7]. Benign lymphoproliferation is found in 40 to 50% of the cases, often presenting as splenomegaly or lymphadenopathy[1]. In the case of lymphadenopathies, they are usually localized in the cervical, mediastinal or abdominal areas. Nevertheless, other tissues such as the liver and gastrointestinal tract are also frequently affected. Non-caseating granulomas, suggesting sarcoid-like changes[2], can be found in lymphoid and non-lymphoid tissues, and are estimated to occur in 8–22% of CVID patients, most frequently affecting the lungs, spleen, liver and lymph nodes[1,5–7,12,13].

In 10% of CVID patients there is liver disease and abnormal hepatic function tests[1]. Nodular regenerative hyperplasia, was reported in 6% of CVID patients and represents the most common liver disease in CVID[14]. Seronegative and granulomatous hepatitis are other entities found in these patients[1].

Disturbances in the gastrointestinal tract are present in up to half of CVID patients, typically reporting clinical manifestations of diarrhea or malabsorption[7]. Possible underlying problems are nodular lymphoid hyperplasia found in 8% of the patients[11], inflammatory bowel disease and higher risk of infection especially by *Giardia lamblia*, followed by *Salmonella* and *Campylobacter jejuni*. Moreover, *Helicobacter pylori* infection should also be considered, as it is present in 80% of patients with CVID who have dyspepsia[2].

Several studies showed that in CVID there is a 1 to 8-fold increase in the risk for overall cancer, being lymphoma the most common malignancy with an increased 12-fold risk, especially non-Hodgkin B-cell lymphoma, followed by a 10-fold increase in risk for stomach cancer, as compared to general population[15].

The current overall life expectancy for CVID patients is over 50 years[1]. Interestingly, autoimmune conditions, cancers other than lymphoma, history of splenectomy, granulomatous disease or the development of bronchiectasis alone have not been associated with reduced survival. In contrast, gastrointestinal disease, liver disease, lymphoma, chronic lung disease or malabsortion were associated with increased mortality[3]. The lower baseline IgG and lower frequency of circulating B-cells were also associated with poorer prognosis[1]. Lung involvement, in particular, is the main cause of death in CVID patients.[3,7]

The immunological studies show that most patients have normal or slightly reduced numbers of peripheral blood B-cells, very low number of plasma cells in the bone marrow, lymph nodes and gut, reduced numbers of memory B-cells (identified by the surface expression of CD27) and reduced numbers of **isotype-switched memory B-cells $CD27^{+}IgD^{-}IgM^{-}$ (SMB)**[1,5–7,16]. SMB develop in the germinal centers of lymph-nodes, in a T-dependent-manner, and therefore their reduction may result from functional defects in either B or T helper cells and correlates with levels of immunoglobulin secretion[17]. A severe reduction in SMB cell type was associated with a greater risk of granulomatous disease and splenomegaly[16]. **Transitional B-cells $CD38^{++}$ IgM^{++} (TR)** are early bone marrow (BM) emigrants which do not proliferate, but instead differentiate into naïve mature B-cells in healthy controls. However, in some CVID patients TR B-cells frequency is increased and, being the most immature form of B-cells detectable in peripheral blood, this probably represents a block in the early

differentiation of mature B-cells[7,18]. TR B-cells expansion was associated with higher risk of lymphadenopathy[16]. Additionally, the expansion of the **$CD21^{low}CD38^{low}$ B-cells (CD21lo)** subset, which is very infrequent in healthy individuals, characterized by low CD21 expression, has been associated with splenomegaly and autoimmune cytopenia[16,18,19].

Besides imbalances in B-cell subsets, CVID is frequently associated with T-cell abnormalities such as persistent T-cell activation, loss of naïve CD4 T-cells and impairment of regulatory T-cells (Treg), which are reported even in patients receiving replacement therapy with IgG[5,7,20,2122,23]. The significant reduction of naïve CD4 T-cells in CVID patients could be explained by both reduced thymic output and increased spontaneous apoptosis and cell turnover[22]. In contrast to CD4 T-cells, the CD8 T-cells may expand, explaining the inverted CD4/CD8 T-cell ratio often seen in CVID[1].

It is not clear which of the T and B-cells abnormalities are possibly causative, and which are secondary or only epiphenomena[24].

Several attempts have been done to establish correlations between immune and clinical phenotypes in CVID. In 2002 the Freiburg classification divided patients into three groups through the analysis of the frequency of different B-cell populations, based on the expression of IgM, IgD, CD27 and CD21[1,25]. Other classification scheme was the Paris classification, which distinguished patients based on the reduction of total versus switched memory B-cells. Thereby, in order to unify different classification schemes and improve their clinical and immune phenotype correlations, the EUROclass classification was developed in 2008. Additionally, immunological classifications based on the underlying pathophysiologic B-cell background were suggested in the past to stratify CVID patients[18]. Notably, none of the models was able to predict autoimmune phenomena[16] and the overall clinical prognosis still imposes a major challenge[18].

Despite recent progress in the discovery of different gene defects associated with CVID, these are considered to cause only 10-20% of the cases in large cohorts[26], and frequently in circumstances of consanguinity, suggesting that this syndrome results from polygenic rather than monogenic causes[5,7]. The key genetic mutations in CVID affect the inducible co-stimulator gene (ICOS)[1,2,27–30], genes that encode for B-cell antigen receptor associated complex (CD19, CD21 and CD81)[1,2,30–34], B-cell activating factor receptor (BAFF-R)[2,33,35], CD20[36], and the BCR transmembrane activator and calcium-

modulating cyclophilin ligand interactor (TACI)[1,2,29,30,33,35]. However, mutations in TACI should be considered as disease modifying rather than disease-causing, because they are not specific of immunedeficient patients[1,2,6,7].

Nowadays the gold-standard treatment for CVID patients is immunoglobulin replacement therapy (IgG), which is known to reduce the frequency of infections and the progression of some complications[2,37]. IgG is administered regularly to patients either by intravenous or subcutaneous infusion. The usual dosage for intravenous immunoglobulin (IVIg) replacement is 100–150 mg/kg/week and 100 mg/kg/week for subcutaneous immunoglobulin, nevertheless individual dosage is usually defined according to maintenance of a target serum immunoglobulin (IgG trough level of at least 500-700mg/dl) and/or to prevent invasive or aggressive infection[1,2,38,39]. Recently, it has been hypothesized that the benefits of IgG therapy are not only related to antibody replacement but also to its ability to modulate the immune response[37]. In addition, management of these patients includes adequate infection control with antibiotic and treatment of eventual complications.

The immunologic and clinical heterogeneity of CVID hampers the discovery of underlying disease-causing mechanisms, genetic defects and clinical relevant prognostic factors.

This study aims to characterize the clinical and laboratory phenotype of CVID patients through the analysis of the largest Portuguese cohort followed in one single Centre in 2015. Additionally, we aim to address the correlations between immune-phenotype and clinical manifestations and to evaluate their stability in CVID patients. For this last objective we focus our analysis in a subgroup of 29 patients, from whom we have detailed clinical and immunological evaluations performed in 2008[35,40,41].

Materials and Methods

Study Design

A total of 60 adult (36 females) CVID patients, 58 of which currently under follow-up in Centro de Imunodeficiências Primárias (CIDP), from Hospital de Santa Maria, were included in this study. Two females were deceased at the time of the study (2015). The patients were diagnosed according to European Society for Immunodeficiency (ESID) criteria[101] and/or to Primary Immunodeficiency Expert Committee (PID EC) of the International Union of Immunological Societies (IUIS), which stated that the criteria for the diagnosis of CVID are based on the severe reduction in at least 2 serum immunoglobulin isotypes (low IgG and IgA and/or IgM), with normal or low number of B cells, and presence of variable clinical phenotypes: most have recurrent infections, some have polyclonal lymphoproliferation, autoimmune cytopenias and/or granulomatous disease[42].

Clinical and immunological data from 60 CVID patients were collected and filled in an online database specifically designed for PID/CVID patients. All the data were then analyzed and the correlations between immune phenotype and clinical manifestations were performed.

All subjects gave written informed consent for blood sampling and processing. The study was approved by the Ethical Board of the Faculty of Medicine of the University of Lisbon and of the Hospital de Santa Maria, and performed in accordance with 1964 Declaration of Helsinki and its later amendments.

Data collection

Clinical and epidemiological data from the 60 patients were assessed retrospectively through the rigorous consultation of the medical records. The information collected regarding each patient included the date of birth, date of the first study, date of the last medical record, duration of follow-up, age/type of first symptom suggesting PID, age at the diagnosis, the age at the beginning of treatment, the frequency of treatment with

antibiotics in the previous year, the current weight, the type/dose of replacement treatment with immunoglobulin G (subcutaneous or endovenous) and other therapeutics required. Furthermore, the delay of diagnosis was calculated based on the difference of the age at first symptoms and the age at diagnosis. The duration of follow-up resulted from the difference between the first medical record and the date of the last medical record. The dose of IgG was calculated in mg/kg/month.

Regarding the current clinical phenotype, it was described if each patient presented autoimmune disease (AID), autoimmune cytopenias, splenomegaly, adenopathies, evidence of lymphoid proliferation, chronic diarrhea, malignancies, bronchiectasis and infections only.

The **criteria for diagnosis** were as follows:

Autoimmune disease - included psoriasis, thyroiditis, vitiligo, Crohn's disease, celiac disease, seronegative arthritis, rheumatoid arthritis, Sjögren's syndrome, SLE, type 1 diabetes *mellitus* and alopecia total. Concerning autoimmune cytopenias it was identified ITP, AIHA, autoimmune neutropenia, autoimmune leucopenia, pernicious anemia and Evans syndrome. Noteworthy, the diagnostic criteria for autoimmune disease, were mainly clinical data, given the impairment in antibody production.

Lymphoid proliferation - it was documented if at least one of the following histologic patterns were present: gastrointestinal lymphoid infiltrate, lymphoid interstitial pneumonitis (LIP) or granulomatous disease on gastrointestinal, lymph node or pulmonary biopsies. Biopsies were performed in 51 patients.

Splenomegaly - longitudinal spleen diameter superior to 13 cm by computer tomography or ultrasonography.

Adenopathies - report of lymph node larger than 1 cm diameter in 2 or more lymphatic chains in clinical and/or imaging exams.

Chronic diarrhea – clinically or laboratory findings of chronic or recurrent diarrhea.

It is important to take in consideration that each individual might have more than one complication.

The **immunological characterization** was based on results of B and T-cell subsets immunephenotype using 8-colour flow-cytometry, available in the files of 52 patients.

These data were compared with 15 age-matched healthy individuals. The laboratory data results quantified the number of total lymphocytes and the absolute number and the percentage of CD19, CD3, CD4, CD8 and NK populations. Regarding the subset of B lymphocytes, it was determined the percentage of naïve cells ($CD27^-$), marginal zone cells ($CD27^+IgD^+IgM^+$), switched memory B cells ($CD27^+$ IgD^-), transitional B cells ($CD38^{hi}IgM^{hi}$), switched plasmablasts ($CD38^{hi}IgM^-$), and $CD21^{low}CD38^{low}$ subset. Concerning T CD4 lymphocytes, it was determined the percentage of naïve ($CD45RA^+CD27^+$) and activated ($HLADR^+$; $CD38^+HLADR^+$; $CD69^+$) cells. In addition, regarding the T CD8 population, the percentage of naïve ($CD45RA^+CD27^+$) and terminally differentiated cells ($CD45RA^+CD27^-$) were also included in the study. Based on B-cell analysis each patient was classified according to the EUROclass categorization.

Data analysis

The statistical analysis was performed using GraphPad Prism 5®, version 5.03 for Microsoft Windows®. Given the non-normal distribution of the data, the non-parametric Mann–Whitney test was performed for group comparisons. Results are expressed as mean ± standard error of the mean and median, and were considered significant at a p-value <0.05.

We compared the immunological parameters in CVID groups split according to the presence of a given clinical manifestation and with a healthy cohort (n=15). We further focused our analysis in a subgroup of 29 patients from whom we have detailed clinical data collected during a previous study (2008)[13,35,40].

Results

The analysis of medical records allowed us to characterize the CVID cohort under follow-up regarding different epidemiological and clinical variables (Suplementary Table 1).

Demographic data

The **age** of the patients ranged from 21 to 78 years, with a mean of 44.6 ± 12.7years old. The **age at onset of symptoms** ranged from 1 to 60 years and was most commonly during the second decade (mean 18.4 ± 15.4years; median 18) and mean **age at diagnosis** was 32.3 ± 14years (median 32.5), with no significant differences between gender, regarding the onset of symptoms and age at diagnosis (p=0.981 and p=0.457, respectively*)*.

The mean **diagnostic delay** was 13.9 ± 12.3years (median 10.5).

Length of follow-up and overall mortality

This cohort had a median follow-up time of 8.5 years. Of the total 60 individuals, two females were deceased at the time of the present study, both of them died from solid malignancies, specifically triple negative breast carcinoma and gastric adenocarcinoma at the age of 43 and 40 years old, respectively.

Initial symptoms and diagnosis

In most patients **the initial symptoms** were recurrent respiratory infections (63%) and in 18% were lower respiratory tract infections. In 32% of the patients, the initial symptom was chronic diarrhea. AID was present as first manifestation in 12% of the total cohort, corresponding to autoimmune cytopenia in 86% of these, specifically ITP. Three patients presented severe infections, namely meningitis and poliomyelitis as first

manifestations (at the ages of 3 and 6 months, respectively), two patients had urinary tract infections and one had recurrent abscesses.

We compared the **mean age at diagnosis** of the patients that present of each clinical manifestation, with the mean age at diagnosis of the individuals who do not and, with mean age at diagnosis of total CVID. Remarkably, there was no significant difference in the mean age at diagnosis of patients first presenting with respiratory infections as compared to the rest of the patients. Among different clinical manifestations, our data shows that the age of diagnosis of patients presenting with splenomegaly (mean 28.9 ± 13.2years, median 29) was significantly lower compared with patients with chronic diarrhea (mean 35 ± 14.3years, median 37), (p= 0.048). There were no other significant differences in the age of diagnosis regarding the different clinical phenotypes.

The **diagnosis delay** ranged from 0 to 44 years, with a mean of 13.9 ± 12.3years (median 10.5). There were no significant differences in the diagnosis delay regarding the different clinical phenotypes.

Clinical manifestations of CVID

The review of the medical records revealed that, as shown in Table 1, at the time of the last medical observation, 34 out of 60 patients (57%) presented **AID**. Specifically, 19 patients presented **autoimmune cytopenias** (32%), including ITP (14), AIHA (5), autoimmune neutropenia (4), Evans syndrome (1), autoimmune leucopenia (1) and pernicious anemia (1). Other autoimmune diseases were found in 21 patients (35%), including psoriasis (5), thyroiditis (7), vitiligo (3), Crohn's disease (3), celiac disease (2), seronegative arthritis (2),

	CVID
Number (male/female)	60 (24/36)
Age (*yrs.*)	45±13
Clinical manifestations[a]	
Infections-only	3/60 (5%)
Autoimmune disease - clinical data, given the impairment in Ab production	34/60 (57%)
Adenopathies- - lymph node larger than 1 cm diameter in 2 or more lymphatic chains in clinical and/or imaging exams	31/60 (52%)
Lymphoid proliferation - diffuse lymphocytic infiltrates on gastrointestinal, lymph node or pulmonary biopsies	43/60 (72%)
Granulomas - granulomas on gastrointestinal, lymph node or pulmonary biopsies	10/60 (17%)
Chronic diarrhea	42/60 (70%)
Splenomegaly- longitudinal spleen diameter superior to 13 cm (computed tomography or ultrasonography)	34/60 (57%)
Malignancy	8/60 (13%)
IgG replacement therapy	
Intravenous	39/56 (70%)
Subcutaneous	17/56 (30%)
Length of IgG therapy (*yrs.*)	11±7
Length of follow-up (yrs.)	8.5

n.a. not applicable, CVID: Common Variable Immunodeficiency Disorders.

[a]Percentage within total cohort evaluated in brackets.

The immunological data from the CVID cohort was compared with an aged-matched healthy cohort (n=15, 10 female; age 39± 11years).

Table 1- Clinical characterization of the cohort.

rheumatoid arthritis-like (2), Sjögren's syndrome (2), SLE (1), type 1 diabetes *mellitus* (1) and alopecia total (1).

Splenomegaly was reported in 34 patients (57%) and **adenopathies** in 31 (52%). Interestingly, 22 patients (37%) showed both simultaneously. Patients 8 and 12 were splenectomized at the age of 51 and 22 years old, respectively, in context of ITP.

Histological evidence of lymphoid proliferation was found in 43 patients, being 72% of the total cohort and 84% among the 51 patients that were submitted to biopsies during follow-up. Interestingly, 1 of the 4 patients that had not been submitted to biopsies in 2008 (patient 56) has gastrointestinal biopsies that present lymphoid proliferation. Moreover, granulomatous disease was documented in ten individuals (17%) and LIP in five (8%). Forty-one patients were documented with gastrointestinal lymphoid infiltrate, mainly nodular lymphoid hyperplasia (20 patients, 33%).

Clinical or laboratory findings of **chronic or recurrent diarrhea** were reported in forty-two patients (72%) and **bronchiectasis** in 35, by chest CT (58%).

Malignancy occurred in 8 patients (13%), including 4 neoplasms from the gastrointestinal tract (3 gastric carcinomas - patients 31, 54 and 59, and 1 rectal carcinoma -patient 41), 3 skin carcinomas (1 squamous-cell carcinoma - patient 15 and 2 basal-cell carcinoma - patients 44 and 58) and 1 breast cancer (patient 6).

Noteworthy, only 3 patients (5%) did not present any complication besides **infections** (not considering bronchiectasis as a clinical phenotype).

Immunoglobulin replacement therapy

Fifty-six patients were currently on regular immunoglobulin replacement therapy with highly variable doses and periodicity, adapted to each patient's weight and clinical condition. The majority (70%) of the cohort was under endovenous IgG, with a mean dose of 629.6 ± 214.5mg/kg/month (median 635). The remaining 30% was under subcutaneous IgG, with a mean dose of 595.4 ± 380mg/kg/month (median 419).

At the time of the study 2 of the 58 patients alive have not started IgG replacement therapy. The age at the beginning of the IgG replacement therapy ranged from 5 to 67

years old with a mean age of 34.4 ± 13.9years (median 36, n=58). The treatment had a mean duration of 10.6 ±6.7 years (median 10) and ranged from 1 to 27years (n=56).

Other therapeutics

Twenty-four individuals were under immunossupressive therapy. Seventeen were under corticotherapy, although only one was being treated with a dose above 20mg/day. Immunossupressive drugs, namely methotrexate, cyclophosphamide and cyclosporine, were being used in four patients for malignancy, granulomatous disease and seronegative rheumatoid arthritis. Infliximab was being used in one patient in the management of seronegative arthritis. Salazopyrin and mesalazine, were in use in nine patients, mainly because of enteropathy, and hydroxychloroquine in one patient with SLE and in another one with RA-like arthritis.

Overlap of the clinical manifestations

Our results show that the majority of the clinical manifestations and/or signs and symptoms do overlap. Regarding the following: autoimmune disease, lymphadenopathies, lymphoid proliferation, splenomegaly, chronic diarrhea and bronchiectasis, we calculated the frequency of different clinical manifestations per patient. We found that each patient presented an average of four of the previous manifestations (mean 3.6 ±1.7; median 4) and that 75% of the patients presented three or more different clinical phenotypes and/or signs and symptoms. In fact, the larger group of CVID patients, present in Figure 1, manifests simultaneously autoimmune disease and lymphoid proliferation (n=21). Regarding signs and symptoms, in Venn diagram in Figure 2, the larger group of patients manifests at the same time adenopathies, diarrhea, bronchiectasis and splenomegaly.

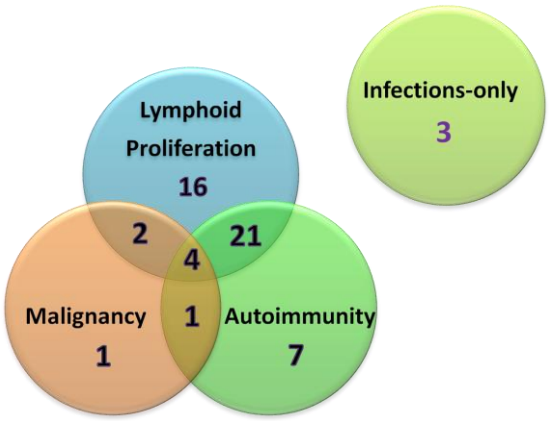

Figure 1 - Venn diagram with the representation of the overlap of the clinical manifestations. DAI - Autoimmune disease.

Figure 2 - Venn diagram with the representation of the overlap of the signs and symptoms.

Stability of the clinical manifestations

In order to evaluate the stability of the clinical phenotype in CVID patients we focused our analysis in a subgroup of 29 patients from whom we have detailed clinical and immunological evaluations performed in 2008[35,40,41]. We compared the current clinical manifestations with the previously recorded phenotypes and the results are shown in Figure 3 and Figure 4.

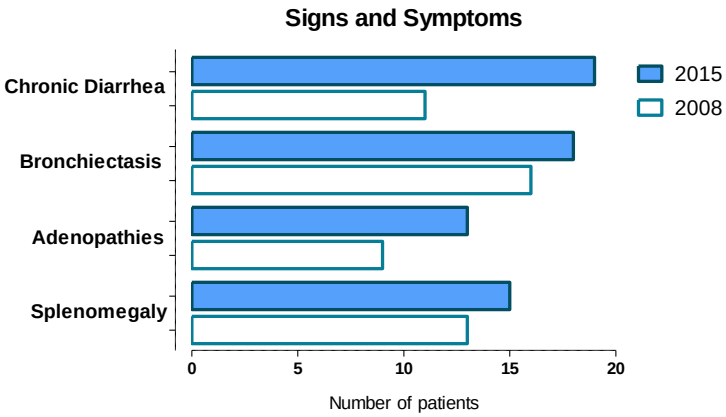

Figure 3- Comparison of the evaluations of signs and symptoms performed in a group of 29 patients in 2008 and 2015.

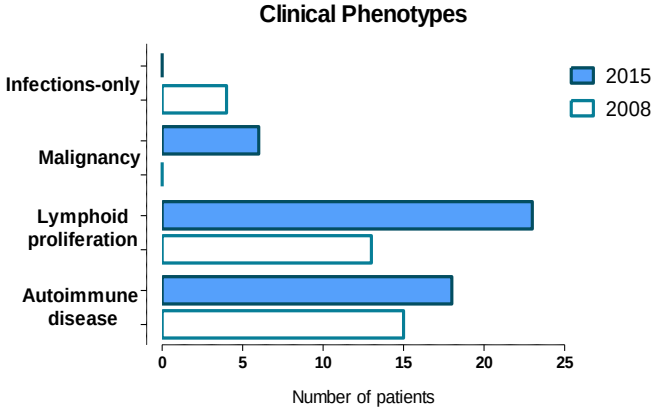

Figure 4- Comparison of the evaluations of clinical phenotypes performed in a group of 29 patients in 2008 and 2015.

We were able to show that the spectrum of clinical manifestations expanded despite replacement IgG treatment. We observed that regarding **autoimmune diseases**, five patients presented cytopenias during the follow-up of seven years, specifically primary immune thrombocytopenia. **Splenomegaly** improved in two cases and was recorded for the first time in four patients, while **adenopathies** cleared in one patient and were first

reported in five. Ten patients revealed *de novo* **gastrointestinal lymphoid infiltrates**, **LIP** was documented in one more patient and **granulomatous disease** in five more patients. Overall, ten more patients present histological evidence of **lymphoproliferation** in 2015. During the follow-up, a **neoplasm** was diagnosed in six patients and **bronchiectasis** in two patients. Finally, eleven patients started to have clinical manifestations of chronic diarrhea and three patients reported improvement of these complains.

Immunological characterization

In 52 patients we analyzed B-Cells and T-cells immune-phenotype, namely in order to evaluate the frequency of the most frequently reported disturbances in CVID in our cohort (Supplementary Table 2).

In contrast with an age-matched healthy cohort, our CVID patients showed decreased frequency of naïve CD4 T-cells (mean 26.4 ± 21.9%, median 20.4), as compared to mean 42 ± 12.3%, median 43.1, (p= 0.0029, Figure 6) and increased immune activation of CD4 T-cells, as evaluated by the frequency of expression of HLA-DR within CD4 T-cells (mean 13.9 ± 10%, median 11.4), as compared to mean 5.4 ± 2%, median 5.5 regarding the healthy cohort, (p= 0.0006, Figure 7).

The CVID cohort presented an expansion of abnormal $CD21^{low}CD38^{low}$ B-cells (mean 18.8 ± 18%, median 11.5), as compared to the healthy cohort (mean 4.1 ± 2.4%, median 3.4), (p=0.0003, Figure 8).

CVID patients have been classified according to the frequency of the above mentioned B-cell sub-populations. The stratification of our CVID cohort according to the **EUROclass classification** is represented in Figure 5[16]. This classification divides the patients in groups with circulating B-cells comprising less than 1% of lymphocytes "B^-" and more than 1% "B^+". Within the "B^+" subset, patients with less than or equal to 2% of switched memory B-cells are grouped into "SmB^-" or "SmB^+", respectively. Additionally, expansion of transitional B^-cells (more than 9%) and CD21lo B-cells (more than 10%) also determine different categories[1,25,43].

Our results revealed no major differences in the distribution of the patients among different Euroclass categories, compared with the results previously reported in the European multicenter trial (n=303)[43], except for the increased relative proportion of $SmB^{+}21^{low}$ patients (p=0.0008).

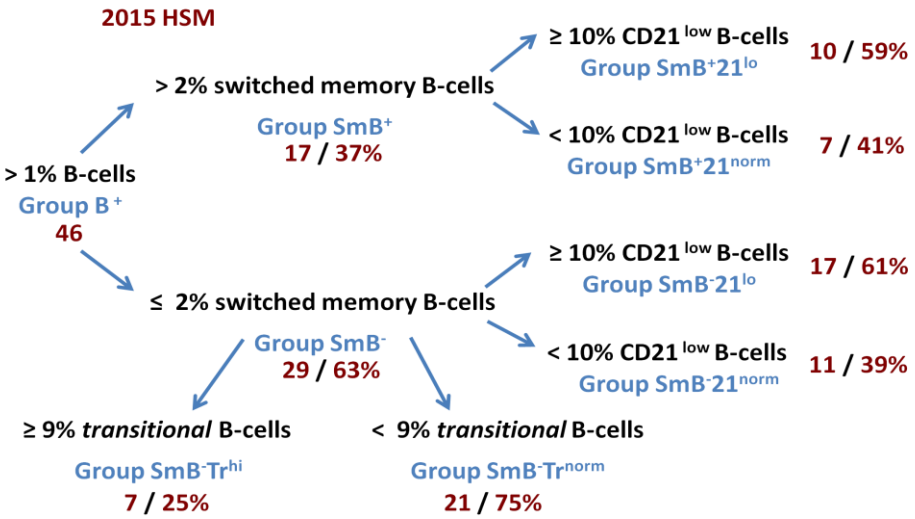

Figure 5- Patients distribution according to EUROclass B-cell subpopulations. The number of patients included in each category and respective frequency in the total cohort are indicated.

Comparison between clinical and immunologic features

We compared the immunological phenotype among CVID groups split according to the presence of a given clinical manifestation (Supplementary Table 3 and Supplementary Table 4) and with a healthy cohort (n=15).

Our results revealed, as shown in Supplementary Table 3, that patients with splenomegaly presented statically lower total number of lymphocytes (mean 1397 ± 1012cells/μL, median 1040) and CD4 T-cell count (mean 585.8 ± 441cells/μL, median 445) compared with CVID patients without this manifestation (mean 1816 ± 612.3cells/μL, median 1840; *p*=0.01 and mean 736.8 ± 316cells/μL, median 69; *p*=0.0351; respectively). Bronchiectasis were present in patients with statically lower number of $CD19^{+}$ cells (mean 6.2 ± 5cells/μL, median 3.8), as compared with patients without this complication (mean 35.45 ± 106.4 cells/μL, median 8.2; *p*= 0.0279). The group of patients presenting chronic diarrhea was not correlated with any immunologic feature.

Patients with autoimmune cytopenias, splenomegaly and adenopathies present significantly higher levels of naïve CD4 T-cell loss (Figure 6). Additionally, significantly higher levels of expression of T-cell activation markers were observed in association with autoimmune cytopenias, splenomegaly, adenopathies and lymphoid proliferation (Figure 7).

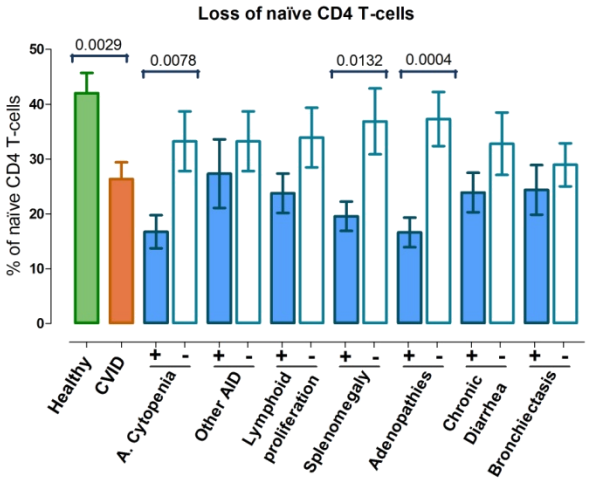

Figure 6 –Loss of naïve T-cells in patients with CVID; comparison between total cohort, healthy individuals, and patients with different clinical phenotypes.

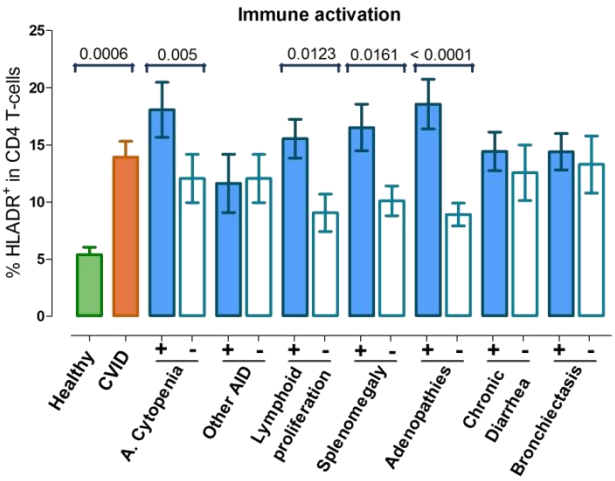

Figure 7- Immune activation in patients with CVID; comparison between total cohort, healthy individuals, and patients with different clinical phenotypes.

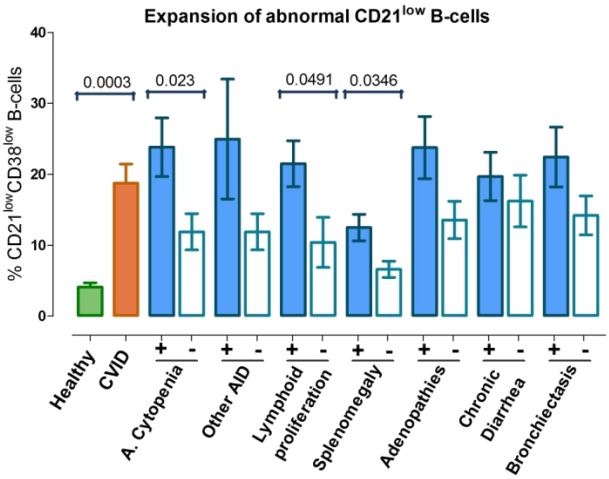

Figure 8- Expansion of of abnormal $CD21^{low}CD38^{low}$ B-cells in patients with CVID; comparison between total cohort, healthy individuals, and patients with different clinical phenotypes.

Moreover, autoimmune cytopenias, splenomegaly, adenopathies and lymphoid proliferation were associated with significant expansion of $CD21^{low}CD38^{low}$ (Figure 8).

Clinical correlations of our cohort **according to EUROclass** categories revealed that the group of patients within $SmB^{-}21^{low}$ group presented higher frequency of autoimmune diseases (p=0.0157), compared with the patients $SmB^{-}21^{norm}$, as well as the individuals $B^{+}SmB^{-}Tr^{norm}$ in comparison with those $B^{+}SmB^{-}Tr^{hi}$ (p=0.0013). Additionally, we also found that patients within $SmB^{+}21^{low}$ group compared with $SmB^{+}21^{norm}$ patients, presented higher frequency of splenomegaly

(p=0.003), whereas those $SmB^{-}21^{low}$ presented higher frequency of adenopathies, when compared with the $SmB^{-}21^{norm}$ individuals.

Discussion

We reviewed epidemiologic, clinical and immunological characterization from a cohort of sixty adult CVID patients (36 females and 24 males) that have been followed in Hospital de Santa Maria (Centro Hospitalar Lisboa Norte), during a mean follow-up time of 8.5 years. The database specially designed for PID/CVID patients and filled with all of the clinical information, regarding the sixty patients, will be used for the medical record of PID patients in the future.

In our study the **onset of symptoms** was most common during the end of the second decade (mean 18.4 ± 15.4years; median 18) which is slightly earlier when compared with the results of other cohorts[3,16,44,45]. In contrast, a more recent European study reported a mean age of onset of 1 to 5 years, which was probably biased by the predominant inclusion of pediatric centers[46].

Regarding the **initial symptoms**, our study reported similar results to the Quinti *et al.* observations' that respiratory tract infections were the most prominent clinical problem at diagnosis, occurring in 65% of the patients[11]. Autoimmune diseases were the first symptoms present in 12% of the cohort, which is also very similar to previous studies[11]. Due to the struggle related to the definition of the first symptoms of CVID, such as the cut off between the normal recurrent respiratory infections expected during the infancy and the recurrent respiratory infections caused by CVID, we compared the difference between the **age at first symptoms** when we take into account or not these manifestations. We calculated the mean age at first symptoms of the total cohort: 1) considering recurrent respiratory infections of the infancy and ascribing the age of 2 years, and 2) considering the following manifestations as the initial presentation of CVID. The results were not significant different (p=0.23) between the consideration, or not, of the respiratory infections during childhood as the first manifestations of CVID to the age of onset (mean 16.2 ±14.9 and mean 19.1±15.1years, respectively).

The average **age at diagnosis** was 32.3 years, which is similar to the reported in the literature[3,11,16,44,45]. The mean **diagnostic delay** was 13.9 years, which is slightly later when compared with previous studies [11,16,45,47], and possibly explained regarding the earlier mean onset of symptoms. Among different clinical manifestations, our data shows that the age of diagnosis of patients presenting with splenomegaly was

significantly lower compared with chronic diarrhea. This finding could probably be explained by the fact that medical doctors correlate more some specific clinical manifestations to immunodeficiency, and tend to be more aware of the diagnosis in those cases.

During follow-up, 55% of the patients presented autoimmune phenomena which is very high compared to the 25-48% documented[3,11,45], nevertheless, as in previous studies, ITP and AIHA were the most common immune cytopenias[3,16]. Chronic diarrhea was observed in 72% of the patients, which is also higher than reported. This difference might be due to the fact that we considered both infectious and non-infectious causes for chronic diarrhea. Furthermore, also splenomegaly, bronchiectasis and gastrointestinal lymphoid infiltrate, mainly nodular lymphoid hyperplasia, were more frequently reported in our cohort compared to what was previously reported in the literature, which could reflect the fact that a lot of manifestations were underdiagnosed until the availability of better diagnostic procedures in patients periodically followed up. Interestingly, two Portuguese studies from 2012 and 2011, that followed a cohort of twenty-four CVID patients in Centro Hospitalar do Porto[48] and fourteen CVID patients in Centro Hospitalar e Universitário de Coimbra[47], respectively, during an identical time of follow-up, had similar results regarding the higher proportion of patients presenting some of the clinical features mentioned, in comparison with studies from other countries. One possible explanation for these results could be related to the similar genetic pool of the Portuguese population or to the fact that patients are only referred to a specialized Centre later in the course of the disease. On the other hand, only 5 %, of the patients did not present any complication besides infections, which is much lower compared with similar studies (26-32%)[3,45,47].

Chapel H. *et al.*has focused the importance of clinical phenotyping, and its value for prognosis, through the definition of 5 distinct clinical phenotypes: no complications, autoimmunity, polyclonal lymphocytic infiltration, enteropathy, and lymphoid malignancy[45]. Moreover, even though, regarding this categorization, 83% from a total of 334 CVID patients had only one of these phenotypes[45], our results showed that by considering clinical manifestations as malignancy, lymphoid proliferation, autoimmunity, infections-only, the majority of the **clinical manifestations do overlap,** which supports the struggle in establishing homogeneous and stable groups of clinical phenotypes[45,49].

Regarding the **correlations between clinical and immunological phenotypes**, we found that autoimmune cytopenias, splenomegaly, adenopathies and lymphoid proliferation were associated with significantly higher levels of naïve CD4 T-cell loss, T-cell activation markers (%HLADR^{+}) and expansion of CD21^{low}CD38^{low} B-cells. In our cohort, we found associations previously reported in EUROclass trial, namely association of expanded CD21^{low} B-cells with splenomegaly and granulomatous disease (lymphoid proliferation)[16] and is also in accordance with the relation with splenomegaly and autoimmune disease, reported by Moratto *et al.*[17]. In addition, our data also resemble the Mouillot *et al.* and Bateman *et al.* results regarding the reduction in naïve CD4 T-cells and increase in activated T-cells in autoimmune cytopenias and lymphoid proliferation[21,22]. Finally, as Lanio *et al.* we also report that lower levels of naïve CD4 T-cells are more frequently observed among patients with splenomegaly[23].

The **longitudinal analysis** of a subgroup of twenty-nine patients demonstrated that none of the clinical manifestations or signs and symptoms were constant findings. In contrast to other studies, we observed an increase in the prevalence of autoimmune cytopenias during follow-up[11]. We show that the spectrum of CVID clinical manifestations in each individual is not stable over the time, expanding despite replacement IgG treatment, and as a result, that CVID is a progressive disease.

We report the usage of slightly higher **dose of IgG in the replacement therapy** in our study, with a median dose of endovenous and subcutaneous IgG of 635 and 419mg/kg/month respectively, compared to the usual dose of 200-600mg/kg/month[38,39]. This difference could eventually be justified by the higher prevalence of autoimmune conditions[44,46]. Moreover, it corroborates the conclusion of a large European cohort of 2212 patients, that there is a considerable difference in the management of CVID among centers and that IgG dosage should be individually based on each patient's clinical course[39,46].

Malignancy is one of the most relevant prognosis factors in CVID[3,11,44]. During the time of follow-up of this study, this cohort was not associated with any case of lymphoma, even though this is the most prevalent malignancy in CVID described in the literature[3,11,15,44]. Nevertheless, we documented seven cases of carcinoma during follow-up and, as expected regarding solid tumors, two of them were gastric carcinoma[3,11,44].

Mortality was low in our cohort (3%), comparable with recent studies with similar follow-up periods (6%)[11]. This reflects the improvement in the management of disease, with more accurate diagnosis and advanced treatment of the disease complications, as well as the more effective IgG replacement treatment protocols.

Our study concludes that although CVID has got as hallmark a severe antibody deficiency, it represents a heterogeneous group of patients with a broad spectrum of clinical and immunological disturbances. **Additional therapies** are required to limit the progression of non-infectious complications that are main determinants of morbidity in CVID patients. Finally, our data also supports that **targeting of T-cell imbalances** may be a promising therapeutic strategy to be explored in the management of these patients in the future.

Acknowledgements

Firstly, I would like to express my gratitude to Professora Doutora Ana Espada Sousa for the motivation to get involved in this project and for providing me with the opportunity to be integrated into A E Sousa Lab/ Laboratório de Imunologia Clínica. I also want to give my sincere gratefulness to Drª Susana Lopes da Silva for the continuous support, valuable guidance and patience given during this research project, as well as the clinical contribute regarding the patients' medical records.

This investigation work could not have been done without Ana Serra-Caetano who helped with the interpretation of immune phenotype studies. I would also like to thank Drª Sara P. Silva for patients' clinical records, that were essential for the present project and for immunological data that were generated in 2008, with Rita R. Barbosa. My sincere thanks also goes to Professor Doutor Manuel Barbosa, for the opportunity of studying this CVID cohort and be part of the research project. Additionally, I am also thankful to all the investigators in A E Sousa Lab for welcoming me in the research unit which made me feel part of the team.

Finally, I want to say a special thank you to my family for their help and endless care and encouragement throughout this journey, to Miguel for the valuable suggestions and support and to my dear friends for the precious times spent together that always make challenges look easier.

All together, the above contributions made it possible to orally present this work and be congratulated with the second prize, during the annual Gabinete de Apoio à Investigação Científica, Tecnológica e Inovação (GAPIC) meeting – Dia da Investigação, and to do a poster presentation during the Grupo Português de Imunodeficiências Primárias (GPIP) conference - VII Reunião De Imunodeficiências Primárias, in Porto.

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SUPPLEMENTARY DATA

Table 1. Patients' clinical profile

Case nr	Gender |Age (y)	Age at 1st symptoms (y) |type	Age at diagnosis (y) |Beginning IgG (y)	AID	Autoimmune Cytopenias	Splenomegaly	Adenopathies	Lymphoid Proliferation	Cancer	Chronic Diarrhea	Bronchiectasis	IGIV/ IGSC |Dose (mg/kg/month)
1	F | 33	2 | RI	27 | 30	ITP	ITP	Yes	Yes	No	No	No	Yes	iv | 729
2	F | 33	1 | RI	10 | 10	No	No	No	Yes	Yes	No	No	Yes	sc | 1442
3	F | 50	35 | RI	39 | 39	Pernicious anemia	Pernicious anemia	No	Yes	Yes	No	Yes	No	sc | 236
4	F | 38	20 | RI	36 | 37	No	No	No	No	No	No	No	Yes	iv | 429
5	F | 47	31 | RI	37 | 37	No	No	No	No	Yes	No	Yes	Yes	iv | 583
6	F | 43†	26 | RI	31 | 31	No	No	Yes	No	No	Yes	No	No	iv | 256
7	F | 35	31|RI; UI; recurrent candidiasis	33 | 33	No	No	No	No	Yes	No	No	Yes	sc | 400
8	F | 60	39 | ITP	52 | 54	Evans Syndrome	Evans Syndrome	No	No	Yes	No	No	No	iv | 577
9	F | 47	20 | RI	38 | 38	Psoriasis; seronegative arthritis	No	Yes	Yes	Yes	No	Yes	No	iv | 370
10	M | 48	25 | RI	31 | 39	No	No	Yes	Yes	No	No	Yes	Yes	sc | 560
11	M | 48	12 | CD	37 | 45	No	No	Yes	Yes	Yes	No	Yes	No	iv | 571
12	F | 32	18 | ITP	29 | 30	Sjögren's syndrome, SLE, ITP, autoimmune neutropenia	ITP, autoimmune neutropenia	No	Yes	No	No	No	Yes	iv | 507
13	F | 37	1 | Meningitis	22 | 22	Celiac disease	No	Yes	Yes	Yes	No	Yes	Yes	sc | 640
14	F | 26	8 | RI	9 | 9	No	ITP	Yes	Yes	Yes	No	No	Yes	sc | 875
15	M | 78	53 | RI	66 | 67	No	No	Yes	No	Yes	Yes	Yes	Yes	iv | 461
16	F | 40	27 | ITP	27 | 27	ITP	ITP	Yes	No	No	No	No	No	sc | 515
17	F | 45	10 | RI	40 | 40	No	No	No	Yes	Yes	No	Yes	Yes	iv | 714
18	M | 44	39 | CD	39 | NA	Psoriasis; Celiac disease	No	No	Yes	No	No	Yes	No	NA
19	F | 71	55 | CD	65 | 66	No	No	No	Yes	Yes	No	Yes	No	iv | 1026
20	F | 36	19 | Febrile syndrome	21 | 27	ITP	ITP	Yes	Yes	Yes	No	Yes	No	iv | 635
21	M | 30	22 | CD; RI	22 |NA	No	No	No	No	No	No	No	No	NA

22	F | 41	2 | CD	37 | 39	No	No	No	No	Yes	No	Yes	Yes	sc | 486
23	F | 40	2 | RI	35 | 35	No	No	No	No	No	No	No	No	iv | 167
24	M | 39	10 |RI	21 | 21	Thyroiditis	No	No	No	No	No	No	No	sc | 400
25	M | 48	2 |RI	41 | 42	Psoriasis	No	Yes	Yes	Yes	No	Yes	Yes	sc | 660
26	M | 28	6 | ITP	9 | 21	ITP	ITP; neutropenia	Yes	Yes	Yes	No	No	Yes	sc | 310
27	M | 72	20 | RI	51 | 51	Vitiligo; Thyroiditis	No	Yes	Yes	Yes	No	Yes	Yes	sc | 390
28	F | 37	15 | RI	17 | 17	AIHA; ITP	AIHA; ITP	Yes	Yes	Yes	No	Yes	Yes	sc | 1600
29	M | 50	38 | RI	39 | 39	No	No	Yes	Yes	Yes	No	Yes	Yes	iv | 702
30	M | 48	21 | recurrent abscesses	33 | 35	No	No	Yes	Yes	Yes	No	No	No	iv | 794
31	M | 46	17 | RI	19 | 19	Psoriasis; Crohn's disease	No	Yes	Yes	Yes	Yes	Yes	Yes	iv | 833
32	F | 36	2 | RI	20 | 20	RA; AIHA	AIHA	Yes	No	No	No	Yes	Yes	iv | 1085
33	M | 57	1 | poliomielitis	22 | 47	AIHA	AIHA	Yes	Yes	Yes	No	Yes	Yes	iv | 667
34	F | 52	2 |CD; recurrent conjutivitis	33 | 39	Vitiligo; Thyroiditis; Sjögren's syndrome; ITP	ITP	No	Yes	Yes	No	Yes	No	iv | 408
35	F | 25	2 | RI	21 | 21	Thyroiditis; DM type 1	No	Yes	No	No	No	Yes	Yes	iv | 741
36	F | 59	2 | RI	46 | 46	No	No	No	No	No	No	Yes	No	iv | 678
37	F | 52	22 | RI	48 | 42	ITP; neutropenia	ITP; neutropenia	Yes	Yes	Yes	No	Yes	Yes	iv | 697
38	F | 47	31 | RI	37 | 37	No	No	No	No	Yes	No	Yes	Yes	iv | 536
39	F | 55	2 | RI; CD	20 | 44	Seronegative arthritis; ITP	ITP	Yes	No	Yes	No	Yes	Yes	iv | 727
40	F | 65	60 |RI	62 | 63	No	No	No	No	Yes	No	Yes	No	iv | 545
41	M | 42	2 | CD	31 | 31	Inflamatory bowel disease	ITP; neutropenia; leukopenia	Yes	No	Yes	Yes	Yes	No	iv | 735
42	F | 38	18 |RI	27 | 27	AIHA; ITP; immune neutropenia	AIHA; ITP; immune neutropenia; leukopenia	No	Yes	Yes	No	Yes	Yes	iv | 1019
43	F | 77	43 |RI	49 | 54	ITP	ITP	Yes	No	Yes	No	Yes	Yes	iv | 896

44	F | 73	32 | ITP	63 | 64	AIHA; ITP; Thyroiditis	AIHA; ITP	No	No	Yes	Yes	Yes	Yes	iv | 538
45	M | 22	3 | RI	12 | 14	No	No	Yes	Yes	No	No	No	No	iv | 441
46	M | 32	27 | RI	30 | 30	No	No	Yes	No	No	No	No	No	iv | 396
47	M | 38	2 |CD	32 | 33	Vitiligo	No	Yes	No	Yes	No	Yes	No	iv | 635
48	M | 48	10 | RI; CD	39 | 40	Crohn's disease	No	No	Yes	Yes	No	Yes	Yes	sc | 416
49	M | 44	1 | Meningitis; tuberculosis	39 | 39	RA-like	No	Yes	Yes	Yes	No	Yes	Yes	iv | 936
50	M | 51	40 | RI	42 | 42	No	No	No	No	Yes	No	Yes	No	sc | 419
51	M | 21	1 | Otitis media	5 | 5	ITP; neutropenia	ITP; neutropenia	Yes	Yes	Yes	No	Yes	Yes	iv | 500
52	M | 43	7 | RI; ITP; AIHA	16 | 38	No	No	Yes	Yes	Yes	No	Yes	Yes	iv | 606
53	M | 39	38 | Celiac disease	38 | 38	No	No	Yes	Yes	Yes	No	Yes	No	iv | 524
54	F | 40†	13| RI; CD	28 | 28	No	No	Yes	No	Yes	Yes	Yes	Yes	iv | 3300
55	F | 40	30 | RI	32 | 31	No	No	No	No	No	No	Yes	No	iv | 426
56	F | 44	29 | RI	31 | 31	No	No	No	No	Yes	No	No	No	iv | 132
57	F | 45	18 | RI	39 | 39	Thyroiditis	No	No	No	Yes	No	Yes	No	sc | 414
58	F | 36	4 | RI	7 | 12	Thyroiditis	No	No	No	No	Yes	Yes	Yes	iv | 678
59	F | 36	16 |alopecia	34 | 18	Total alopecia	No	Yes	Yes	Yes	Yes	Yes	Yes	iv | 909
60	M | 47	21 | CD	21 | 21	Psoriasis	No	Yes	No	Yes	No	No	Yes	sc | 359

† - patient deceased at the time of the study

RI – respiratory infection; UI- urinary infection; ITP – primary immune thrombocytopenia; CD – chronic diarrhea; AIHA – autoimmune hemolytic anemia; RA - rheumatoid arthritis; SLE – systemic lupus erythematosus

NA – not applicable

Table 2. Patients' immune phenotype

Case Nr	Lymph	Total lymph cells/µL	CD19+ %	NK %	CD3 %	CD4 cells/µL	CD8 %	B-cells	$CD27^-$ %	$CD27^+IgD^-$ %	$CD38^{hi}IgM^{hi}$ %	$CD21^{lo}CD38^{lo}$ %	EUROclass	CD4 T-cells	$CD45RA^+CD27^+$ %	$HLADR^+$ %	$CD38^+HLADR^+$ %
1		1970	9,1	3,4	76,5	697	36,3		87,5	0,4	1,2	11,5	$B^+ smB^- Tr^{norm} 21^{lo}$		16	11,2	8,7
2		2400	4,08	6,74	87,9	938,77	47,8		93,42	0,75	3,45	22,18	$B^+ smB^- Tr^{norm} 21^{lo}$		40,1	12	7,79
3		1900	21,5	10,56	67,2	768,63	30,8		82,37	2,34	4,53	3,13	$B^+SmB^+ Tr^{norm;} 21^{norm}$		17,3	6,24	3,49
4		1080	10,6	6,4	77,4	438	31,4		80,3	2	1,8	6,04	$B^+ smB^- Tr^{norm} 21^{norm}$		11	10,5	7,8
5		1400	2,74	3,38	91,1	737,18	40,4		95,83	1,24	7,36	7,41	$B^+ smB^- Tr^{norm} 21^{norm}$		95,7	6,17	3,96
6		1800	9,6	24,99	64,1	740,74	31,2		72,37	1,36	1,68	19,29	$B^+ smB^- Tr^{norm} 21^{lo}$		39,5	6,2	4,82
8		1840	3,3		88,1	949	32,3		97	0,4	13,2	34,3	$B^+SmB^-Tr^{hi} 21^{lo}$		48,5	14,2	9,5
9		1000	14,18	7,84	75,2	495,57	24,3		86,38	3,25	6,27	14,76	$B^+SmB^+Tr^{norm;} 21^{lo}$		20,4	6,22	3,32
10		1100	1,45	8,03	86	323,53	61		88,96	4,81	9,28	1,36	$B^+SmB^+Tr^{hi} 21^{norm}$		54,9	8,04	4,74
11		510	8,1		76	218	28,1		83,9	1,6	0,2	10,7	$B^+ smB^- Tr^{norm} 21^{lo}$		0,9	51,7	46,4
13		770	1	5,4	91,9	330	43,3						B^-		22,6	7,8	6,3
14		500	3,42	10,56	83,6	291,35	22,9		84,22	3,8	0,77	10,21	$B^+SmB^+Tr^{norm;} 21^{lo}$		3,61	17,3	12,3
15		1400	10,42	10,42	78,7	444,03	45,6		38,02	11,55	1,06	21,15	$B^+SmB^+Tr^{norm;} 21^{lo}$		9,44	10,5	5,08
16		2000	6,43	5,12	86,8	1009,76	37,8		78,75	2,12	4,6	40,75	$B^+SmB^+Tr^{norm;} 21^{lo}$		8,75	25,8	17,5
18		1870	10.6	1,6	81,7	886	31		77,1	5,8	2,6	7,3	$B^+ smB^+ 21^{norm}$		38,3	5	2,5
19		440	1,9	42,1	49,7	121	20		98,6	1	0	33,9	$B^+ smB^- Tr^{norm} 21^{lo}$		15,7	26,9	25
20		800	5.02	9,27	77,7	518,41	12,3		87,01	0,92	14,18	30,25	$B^+SmB^-Tr^{hi} 21^{lo}$		8,6	30,9	27,1
21		1720	13,4		61,6	506	18,5		92	1,4	0,8	0,7	$B^+ smB^- Tr^{norm} 21^{norm}$		39,4	5,5	3
22		1810	15,9		68,5	643	28,8		33,1	7,7	0,4	6,1	$B^+ smB^+ 21^{norm}$			11,2	8,9
24		1230	0,3	12,2	78,9	139	63,3						B^-		77,5	2,8	1,4
26		600	7,09	7,36	79,1	212,62	48,3		94,16	0,53	12,75	6,07	$B^+SmB^- Tr^{hi} 21^{norm}$		10	36,5	32
27		5400	3,4	26,78	68,6	2037,42	37,3		75,36	0,68	5,16	53,51	$B^+ smB^- Tr^{norm} 21^{lo}$		4,66	31,3	24,4
28		610	3,6	23,2	62,6	263	16,8		99,9	0,1	0	56,6	$B^+ smB^- Tr^{norm} 21^{lo}$		8,6	18,8	16,2
29		650	0,6	4,4	51,4	122	30,5						B^-		21,1	15,5	8,5
30		1749	14	1,8	82	735	36		2,7	<0,1			$B^+ SmB^-$		41	9	5
31		1000	1,81	2,9	92,5	539,28	37		86,82	3,55	4,06	48,19	$B^+SmB^+Tr^{norm;} 21^{lo}$		11,5	14,2	11,1
32		1700	<1	10,88	87,2	421	70,4						B^-		32,1	12	9,57

33		1240	1,5	2	87,7	317	54,1		90,9	0,7	9,2	31,8	$B^{+}SmB^{-}Tr^{hi}21^{lo}$		35,8	16,4	10,7
34		2500	4,35	12,31	81,1	1419,25	70		96,92	0,31	13,01	18,1	$B^{+}SmB^{-}Tr^{hi}21^{lo}$		9,87	20,9	11,9
36		2300	6,32	10,29	81,1	1247,89	30,6		75,66	2,51	1,01	4,52	$B^{+}SmB^{+}Tr^{norm;}21^{norm}$		15,1	8,53	4,16
37		930	6,2		86,6	565	22,8		95,3	0,8	0	13	$B^{+}smB^{-}Tr^{norm}21^{lo}$		4,1	22,1	18
38		1300	3,25	5,5	90,6	619,52	45,4		94,6	0,87	1,98	4,83	$B^{+}smB^{-}Tr^{norm}21^{norm}$		92	6,97	3,7
39		2900	11,01	6,63	82,2	1377,84	38,3		60,93	1,93	4,12	39,54	$B^{+}smB^{-}Tr^{norm}21^{lo}$		21,4	7,95	3,92
40		1960	12,7	5,7	74,8	904	25		83	1,7	3,2	9	$B^{+}smB^{-}Tr^{norm}21^{norm}$		46,2	5,1	3,6
41		700	10,76	4,03	81,6	470,67	13,1		82,51	2,05	4,09	21,62	$B^{+}SmB^{+}Tr^{norm;}21^{lo}$		20,4	6,22	3,32
42		1900	6,28	4,7	88,4	694,82	31,8		93,82	0,29	13,21	35,4	$B^{+}SmB^{-}Tr^{hi}21^{lo}$		4,11	12,3	10,3
43		600	2,74	7,5	84	352,3	17,5		95,16	1,01	32,59	2,85	$B^{+}SmB^{-}Tr^{hi}21^{norm}$		24,1	11,6	7,42
44		2100	5,92	13,27	78,9	845,02	44,7		88,59	1,06	0,22	12,32	$B^{+}smB^{-}Tr^{norm}21^{lo}$		9,78	15,7	9,88
46		2790	8,2	2,4	86,7	840	48,6		67	1,5	7,8	7,7	$B^{+}smB^{-}Tr^{norm}21^{norm}$		22,9	12,4	7,7
47		1040	10,4	1,2	82,6	445	33,8		94,3	0,5	3,3	0,5	$B^{+}smB^{-}Tr^{norm}21^{norm}$		28,9	10,8	6,3
48		3400	15,86	15,44	67,4	621,02	63,1		94,87	0,24	6,38	3,3	$B^{+}smB^{-}Tr^{norm}21^{norm}$		16,9	15,4	6,84
49		700	10,2		78,4	145	53,4		97,4	0,9	5	0,9	$B^{+}smB^{-}Tr^{norm}21^{lo}$		14,8	24,3	20,4
50		1600	4,91	11,57	80,2	541,51	31,2		94,15	1,46	5,25	8,46	$B^{+}smB^{-}Tr^{norm}21^{norm}$		27,1	7,33	3,99
51		2480	0,8		92,9	1433	29,2						B^{-}		5,3	38,5	30,7
52		1830	17,6		80,1	937	25		97,7	0,1	0,1	45,3	$B^{+}smB^{-}Tr^{norm}21^{lo}$		6	13	11,7
53		1550	5,3	6,6	82,9	330	36,5		76,3	2,6	2	10,3	$B^{+}smB^{+}21^{lo}$		14,5	16,3	9
54		1800	<1	5,47	90	1163,16	25,7						B^{-}		38,9	2,95	1,85
55		1480	7,9		84,2	696	29,3		96	0,3	1,4	0,4	$B^{+}SmB^{+}Tr^{norm;}21^{norm}$		49	4,8	2,7
56		2400	8,27	10,45	78,1	1128,39	32,6		71,15	8,2	6,53	8,29	$B^{+}SmB^{+}Tr^{norm;}21^{norm}$		47,2	9,64	5,59
58		1500	11,34	6,23	80,1	629,59	41,6		60,63	2,17	0,04	14,88	$B^{+}SmB^{+}Tr^{norm;}21^{lo}$		36,6	5,11	2,77
59		480	2,2	4,8	86,5	284	26		82,1	7,1	0,2	78,6	$B^{+}smB^{+}21^{lo}$		2,2	13,8	11,9
60		700	3,41	23,51	66,7	101,32	74,6		72,86	3,5	0	27,61	$B^{+}SmB^{+}Tr^{norm;}21^{lo}$		53,7	2,93	1,04

Lymph – lymphocytes

Table 3. Comparison of clinical and immunological phenotypes

Clinical Phenotypes	Total lymph cells/µL	$CD19^+$ %	NK %	CD3 %	CD4 %	CD4 cells/µL
Other autoimmune disease	ns	ns	ns	ns	ns	ns
Autoimmnune Cytopenia	ns	ns	ns	ns	ns	ns
Splenomegaly	**0.0103**	ns	ns	ns	ns	**0.0351**
Adenopathies	ns	ns	ns	ns	ns	ns
Lymphoid proliferation	ns	ns	ns	ns	ns	ns
Chronic diarrhea	ns	ns	ns	ns	ns	ns
Bronchiectasis	ns	**0.0279**	ns	ns	ns	ns

Significant differences regarding lymphocytes subpopulations are indicated for each clinical phenotype.

Each value indicated in the table represents the *p*-value

ns – not statistically significative; lymph- lymphocytes

Table 4 - Comparison between clinical and immunological phenotypes

Clinical Phenotypes	B-cells	$CD27^-$ %	$CD27^+IgD^-$ %	$CD38^{hi}IgM^{hi}$ %	$CD21^{lo}CD38^{lo}$ %	CD4 T-cells	$CD45RA^+CD27^+$ %	$HLADR^+$ %	$CD38^+HLADR^+$ %
Other autoimmune disease		ns	ns	ns	ns		ns	ns	ns
Autoimmune cytopenia		ns	ns	ns	**0.023**		**0.0078**	**0.0053**	**0.0063**
Splenomegaly		ns	ns	ns	**0.0346**		**0.0132**	**0.0161**	**0.0149**
Adenopathies		ns	ns	ns	ns		**0.0004**	**<0.0001**	**0.0001**
Lymphoid Proliferation		ns	ns	ns	**0.0491**		ns	**0.0123**	**0.0237**
Chronic Diarrhea		ns	ns	ns	ns		ns	ns	ns
Bronchiectasis		ns	ns	ns	ns		ns	ns	ns

Significant differences regarding lymphocytes subpopulations are indicated for each clinical phenotype.

Each value indicated in the table represents the *p*-value.

ns – not statistically significative